THE ISOLATION OF THE FUNGUS THAT CAUSES CITRUS MELANOSE AND THE PATHOLOGICAL ANATOMY OF THE HOST [1]

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INTRODUCTION

Citrus melanose [4] is characterized by the presence of small, pustular lesions that look like drops of caramelized sugar on leaves, twigs, and fruits. These lesions are initiated while the parts are still young, and at maturity they may occur as isolated dots or may be arranged in streaks, rings, or extensive irregular patches. The fungus that causes melanose possesses two stages, a pycnidial one, *Phomopsis citri (2)*,[5] described in 1912, and an ascigerous one, *Diaporthe citri (10)*, described in 1926.

Melanose was first described in 1896 by Swingle and Webber *(8)*, who first observed it in November, 1892, at Citra, Fla. They were unable to establish the cause definitely, but assumed it to be a fungus. Furthermore, until now, no one has succeeded, even after repeated attempts, in isolating the causal organism from melanose lesions. The pathogene has been isolated repeatedly, however, from dead twigs and from fruits affected with stem-end rot, two manifestations of the disease that can not properly be designated melanose, but that result from infection by the same organism. The relationship between stem-end rot and dying back of twigs has been determined by the investigations of Fawcett *(2)* and the relationship of melanose to the other two manifestations by Stevens *(7)* and by Floyd and Stevens *(4)*.

Fawcett *(2)* isolated *Phomopsis citri* from the interior of fruits affected with stem-end decay and from the interior of dead twigs. He showed by the cultural similarity of isolations from these two sources and by inoculations that these two forms of the disease are caused by the same fungus.

Floyd and Stevens *(4)*, who did not suspect at first that melanose and stem-end rot were related, later found that the two forms were undoubtedly caused by the same fungus. They occasionally found particles of fungous hyphae in melanose lesions, but were not able to demonstrate whether these were parts of the causal organism or of some secondary invader. They concluded from their microscopic examination of lesions of different ages that no bacterial or fungous organism that could be considered a cause of the disease could be found connected with the affected tissues or the adjoining cells. They tried to isolate the causal organism by planting on various media bits of

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diseased leaves that had been soaked in 1:1,000 mercuric chloride for three to five minutes and washed in sterile water. In nearly every case Colletotrichum overran the cultures and made it impossible to isolate any other fungus that might have been present.

Stevens (7) in 1918 concluded that there was no growth of the fungus within the affected tissues. All attempts to isolate the fungus from artificial inoculations gave negative results, and the organism was never obtained from spots or markings formed naturally. Melanose infections were secured, however, from inoculations with pure cultures of *Diaporthe citri.*

Winston, Bowman, and Bach (9) made fully 1,000 systematic attempts to recover the causal organism from melanose blemishes, but without success in a single instance. Leaf and fruit tissues from both old rough lesions and young, almost invisible spots were cultured. Such surface disinfecting agents as ethyl alcohol, ether, acetic acid, mercuric chloride, and hydrogen peroxide were used, after which the material was rinsed in sterile tap water before being planted in cultures. Repeated attempts were also made to isolate the organism without first subjecting the lesions to surface disinfection.

While the results of previous investigations of melanose, stem-end decay, and the dying back of twigs and branches have left no reasonable doubt as to the identity of the fungus that causes them, the failure to isolate the pathogene from melanose lesions has made it impossible to fulfill completely Koch's postulates. The present study is, therefore, concerned both with the isolation of the pathogene from melanose lesions and with the pathological anatomy of the host, to which no special attention has been given except by Floyd and Stevens (4). The results show that it is possible to complete the rules of proof of pathogenicity of the fungus that causes melanose, and the findings and interpretations relative to anatomical changes that are herein recorded are believed to contribute further to an adequate understanding of the disease.

ISOLATION

Young artificially inoculated orange leaves were used in the preliminary trials. These leaves had been inoculated eight days previously with suspensions of conidia from pure cultures. The young lesions, which were plainly visible at this time, were excised, dipped in 95 per cent alcohol, flamed, and placed on slants of 2 per cent potato-dextrose agar. The resultant mycelial growth in 7 of the 10 slants presented the characteristic appearance of *Diaporthe citri,* while no growth occurred in the other three tubes. This was so unusual in the light of previous experience that subcultures were made on sterilized stems of pigeon pea (*Cajanus indicus*), a substratum favorable for pycnidial formation, in order to induce the development of fruiting bodies. Conidia that were typical of the pycnidial stage, *Phomopsis citri,* were produced in due time in these subcultures. Additional proof of this identity was secured by the inoculation of young grapefruit leaves with these cultures. Infection resulted and typical melanose markings developed.

As a consequence of the successful isolation of the pathogene in this preliminary trial, further attempts were made to obtain the melanose fungus in culture from lesions of varying ages on leaves,

twigs, and fruits.   In all cases the surfaces of the affected parts were disinfected by dipping them in 95 per cent alcohol and removing the alcohol by flaming.   The lesions were then excised and placed on agar plates or slants.   As soon as growth had proceeded sufficiently, which usually required four or five days, subcultures were made from portions of the colonies that looked like the melanose fungus.   In a number of cases, especially when young lesions were used, the fungus appeared in pure culture in the planted plates or tubes and it was not necessary to make subcultures.   As a routine practice, however, the fungus can not be isolated without making subcultures so as to separate the pathogene from the various secondary invaders that early overgrow the cultures.   It is possible that the failure of other investigators to isolate the melanose organism is due in part at least to their failure to use subcultures, and in consequence the causal fungus was crowded out or intermingled with those fungi that grew more rapidly.

In order to test the efficacy of this method of surface disinfection, two normal grapefruit leaves were immersed on June 29, 1926, in a suspension of conidia of the Phomopsis stage.   After they had dried one was dipped in alcohol, flamed, and bits of the leaf tissue were planted on agar.   Fragments of tissue from the other were planted without disinfection.   Of six plantings made from each leaf no growth appeared in any from the first leaf, while three cultures of the melanose organism and three of other species of fungi were obtained from the second.   This test was repeated on July 12, 1926, when six plantings were again made from a disinfected leaf and six from one that was not given surface disinfection.   Again no growth appeared in the first case, and the second yielded two cultures of *Diaporthe citri* and four of other organisms.   A third trial made in the same manner on March 18 employed 20 plantings of leaves that were given surface disinfection and an equal number that were not disinfected. No fungous growth appeared around any of the plantings from disinfected leaves, whereas 14 cultures of the melanose fungus were recovered from those that were not disinfected, 5 yielded other fungi, and the other remained sterile.   This method of surface disinfection is therefore regarded as effective, at least for comparatively normal leaf tissue.   That it is also highly effective for tissues with minute fissures is indicated by the results given later.   (Table 3.)

During the course of this investigation several additional tests of the effectiveness of this method of surface disinfection were made, employing leaves and twigs that were free from melanose lesions and apparently normal but on the surface of which the conidia may reasonably be presumed to have been present.   The results are summarized in Table 1.

No cultures of the melanose organism were secured in this series of 108 trials, although 4 yielded *Colletotrichum gloeosporioides*, a fungus that appears always to be present on citrus throughout Florida.   It seems improbable that the conidia of this Colletotrichum would survive disinfection and at the same time those of the melanose fungus be destroyed.   It may be that the tissues were only apparently normal and that the fungus had established itself in minute fissures.

TABLE 1.—*Results of experiments to isolate Diaporthe citri from apparently normal tissues*

Source of material	Date of experiment	Number of plantings made	Number of plantings yielding—		Number of plantings remaining sterile
			Diaporthe citri	Miscellaneous organisms only	
Old orange leaves	Oct. 31, 1925	9	0	0	9
Mature grapefruit leaves	----do	6	0	0	6
Leaves of Chaetospermum glutinosum	Nov. 7, 1925	6	0	0	6
Mature orange leaves	----do	14	0	1	13
Leaf scars on orange twigs	Nov. 15, 1925	15	0	0	15
Leaves of oranges from June flush	June 15, 1926	16	0	1	15
Orange twigs of spring growth	June 19, 1926	12	0	0	12
Old grapefruit leaves	July 12, 1926	11	0	0	11
Mature grapefruit leaves	June 15, 1927	19	0	2	17
Total		108	0	[a] 4	104

[a] Colletotrichum gloeosporioides.

The attempts to isolate *Diaporthe citri* from melanose lesions have extended over a period of three seasons. Use has been made of lesions on leaves, twigs, and fruits of orange and grapefruit, on leaves of tabog (*Chaetospermum glutinosum*), on twigs of Mexican lime, and on fruits of faustrime (Mexican lime × Australian finger lime). Table 2 contains a summary of the essential features and results of these isolation experiments.

A total of 115 cultures of the melanose fungus have been obtained from 506 plantings of melanose lesions. These lesions varied in age from 6 days to approximately 9 months. Those of definitely known age were obtained by artificial inoculation or occurred following rains on April 8, 1926, and February 14, 1927. The age of the other natural infections was estimated from the age of the flush of growth on which they occurred.

It is of special interest to note, too, that cultures from lesions that had not yet advanced to the stage in which the cuticle had become fissured yielded the pathogene in pure culture or else remained sterile. In general, a larger proportion of successful attempts resulted from isolations from young lesions than from old ones, since various other fungi, primarily *Colletotrichum gloeosporioides* and secondarily *Diplodia natalensis*, were always present in mature lesions. In no case was the percentage of successful attempts to isolate *D. citri* from melanose lesions as large as usually results from attempts to isolate other plant pathogenes from other host tissues. One probable reason for the relatively small number of successful attempts to isolate the melanose fungus is, as will be shown subsequently, that the infected tissues are flooded with gum, which may envelop the mycelium and prevent it from growing out of the tissues in culture. Furthermore, as will be shown in this paper, anatomical studies reveal the fact that the host cells are disintegrated by enzymes. A concomitant digestion of the hyphae of the pathogene may therefore be expected.

TABLE 2.—*Results of experiments to isolate Diaporthe citri from melanose lesions*

Approximate age of lesions	Source of material	Type of infection	Date of experiment	Number of plantings made	Number of plantings yielding—		Number of plantings remaining sterile
					Diaporthe citri	Miscellaneous organisms only	
6 days	Young grapefruit leaves	Artificial	May 23, 1926	6	2	0	4
Do	do	do	June 15, 1926	15	4	2	9
8 days	do	do	Oct. 17, 1925	10	7	0	3
10 days	Grapefruit leaves	Natural	Feb. 24, 1927	24	5	7	12
15 days	Orange twigs	Artificial	July 12, 1926	8	1	0	7
Do	Grapefruit leaves	do	July 27, 1926	11	4	3	4
21 days	Lime twigs	Natural	June 30, 1927	19	4	0	15
27 days	Young orange fruit	do	May 5, 1926	6	2	1	3
34 days	Lime twigs	do	July 11, 1927	16	8	8	0
Do	Young grapefruit fruit	do	May 12, 1926	15	6	5	4
42 days	Grapefruit leaves	do	July 12, 1926	15	2	1	12
Do	Orange leaves	do	July 16, 1926	12	1	11	0
Do	Leaves of Chaetospermum glutinosum.	do	Oct. 20, 1925	10	2	6	2
44 days	Grapefruit leaves	do	July 22, 1927	23	4	4	15
45 days	Orange fruit	do	May 12, 1926	20	5	11	4
49 days	Grapefruit leaves	do	July 27, 1926	7	1	0	6
63 days	do	do	June 11, 1926	8	2	3	3
Do	Orange fruit	do	do	3	2	0	1
Do	Grapefruit twigs	do	do	6	1	0	5
70 days	Grapefruit fruit	do	Aug. 12, 1926	11	1	10	0
71 days	Orange leaves	do	June 19, 1926	12	3	6	3
80 days	Orange fruit	do	June 28, 1926	6	1	0	5
Do	Grapefruit leaves	do	do	10	4	2	4
84 days	Orange leaves	do	Dec. 17, 1925	6	2	1	3
87 days	Grapefruit leaves	do	June 15, 1927	23	4	1	18
91 days	Orange leaves	do	Sept. 11, 1926	20	7	2	11
94 days	Grapefruit leaves	do	June 22, 1927	22	4	5	13
Do	do	do	July 12, 1926	24	5	7	12
Do	Grapefruit twigs	do	do	9	1	8	0
Do	Faustrime fruit	do	July 16, 1926	8	1	4	3
103 days	Orange leaves	do	Dec. 11, 1925	6	2	1	3
130 days	Grapefruit fruit	do	Aug. 16, 1926	12	1	11	0
131 days	Orange fruit	do	Aug. 17, 1926	27	3	16	8
149 days	Faustrime fruit	do	Sept. 4, 1926	11	1	5	5
152 days	Orange fruit	do	Sept. 7, 1926	6	0	4	2
167 days	Grapefruit leaves	do	Sept. 22, 1926	8	1	7	0
179 days	do	do	Oct. 4, 1926	18	2	14	2
183 days	do	do	Oct. 8, 1926	10	1	1	8
270 days	do	do	June 11, 1926	13	7	1	5
278 days	do	do	June 19, 1926	10	1	0	9
Total				506	115	168	223

The reisolation of the melanose fungus from infections resulting from artificial inoculation makes it possible to complete, for the first time, Koch's rules of proof of pathogenicity.

Although young lesions yielded the melanose pathogene in pure culture, and its presence in mature lesions is presumably due to the fact that the mycelium remains alive from the time of primary infection, this may not necessarily be the case. Additional evidence on this point comes from the planting of lesions resulting from mechanical injuries and from attacks of the rust mite (*Phyllocoptes oleivorus* Ashm.). The data on these tissue plantings are assembled in Table 3.

TABLE 3.—*Results of experiments to isolate Diaporthe citri from various lesions on grapefruit other than melanose*

Source of material	Date of experiment	Number of plantings made	Number of plantings yielding		Number of plantings remaining sterile
			Diaporthe citri	Miscellaneous organisms only	
Abrasions from rubbing on fruit	May 12, 1926	10	0	0	10
Greasy melanose on leaves of September, 1925	June 11, 1926	10	1	4	5
Abrasions on fruit from contact with limbs	June 15, 1927	25	0	19	6
Russeted fruit from rustmite injury	___do___	20	1	16	3
Russeted fruit	June 22, 1927	21	0	2	19
Abrasions on fruit from rubbing	___do___	22	0	2	20
Abrasions on fruit	June 30, 1927	24	1	9	14
Abrasions on June bloom fruit	July 11, 1927	12	0	12	0
Rust-mite russeted fruit	___do___	12	0	10	2
Russeted fruit	July 22, 1927	21	1	12	8
Total		177	4	86	87

Four of the 177 trials in this series yielded cultures of the melanose fungus, and *Colletotrichum gloeosporioides* again predominated among the miscellaneous organisms present. In the light of the previously mentioned results on the effectiveness of the method of surface disinfection, it seems improbable that these cultures originated from conidia that were present on the surface or were lodged within crevices of the lesions. As is well known, not only Colletotrichum but also the melanose fungus can occupy tissues saprophytically. The dead-twig manifestation of the disease, in the case of the latter organism, is evidence of this condition. It seems more reasonable, therefore, to believe that the colonies resulted from mycelia within the tissues. This is substantiated by microscopic examination of russeted and abraded citrus tissues, which reveal the universal presence of hyphae within them.

PATHOLOGICAL ANATOMY

Collections of material from natural infections that occurred during April and June were used in studying the anatomy of lesions in advanced stages of development. Infections resulting from artificial inoculations with pure cultures were employed in studying the early stages of the disease. The inoculations were made by wetting bits of absorbent cotton in suspensions of conidia and placing them upon tender leaves and fruits of grapefruit. These were then protected against desiccation by being wrapped in waxed paper. Both free-hand and paraffin sections stained in Haidenhain's iron-alum haematoxylin were used. Infection is effected 36 to 48 hours after inoculation by direct penetration of the upper epidermis. This phenomenon can best be observed in free-hand sections cut parallel to the leaf surface. The germ tube penetrates the cuticle and passes downward between the lateral walls of adjacent epidermal cells. (Fig. 1, A.) Thence it branches and extends intercellularly between the palisade parenchyma.
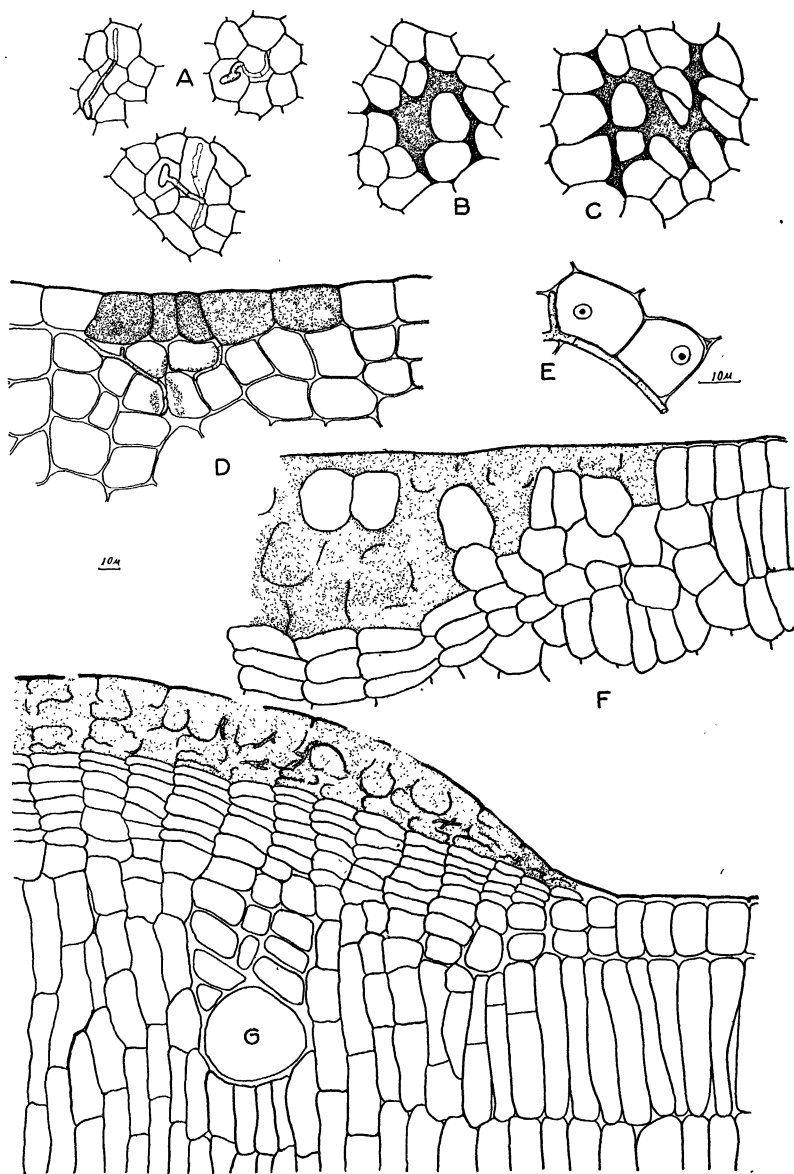
FIG. 1.—A, surface views of grapefruit leaves showing penetration of conidial germ tubes into vertical walls of epidermal cells, 36 hours after inoculation.   B, surface view of a grapefruit leaf bearing a melanose lesion 80 hours old, showing gelatinization of cell walls.   C, lesions on grapefruit leaves 110 hours old, with the dissolution of primary cell membranes, and the resultant formation of free-floating cells.   D, margin of a melanose lesion 80 hours old on young fruit in vertical section.   The center of the lesion is sunken, the cells are collapsed or filled with gum, and the hyphae are intercellular.   E, intercellular hypha of *Diaporthe citri*.   F, vertical section of a lesion 7 days old showing dissolution of cell walls with resultant gummosis and the beginning of the formation of the suberized layer.   G, mature melanose lesion in which the corky layer has completely separated the affected tissues from the subjacent normal tissues.   The cuticle has been ruptured by tensions and the gum mass has become brown.   (A, B, C, D, F, and G were drawn to the scale shown below D, and E to the scale at its right)

There is no evidence of infection visible to the unaided eye until the fourth day after inoculation. At this time the epidermal cells and intercellular spaces are filled with a gummous substance that gives a bright-red precipitate when treated with hydrochloric acid and phloroglucin. This gummous substance is manifestly a hemicellulose derivative resulting from the digestion of the cell wall by pectic enzymes. That such enzymes are present is shown by cultures of the melanose fungus on pectin agar, which was made by the addition of pectin to plain agar. Pectin from two sources was used, a commercial lemon pectin in powdered form and a commercial apple pectin purified by repeated precipitation with alcohol. The initial reaction of the media was adjusted to approximately pH 5, and methyl red was added as an indicator. The color disappeared in a broad area surrounding the colonies, which indicated an increase in alkalinity as a result of the growth of the fungus. A narrow clear zone formed at the borders of the colonies resulted from the digestion of the pectin by enzymes.

It is apparent from examination of lesions four to five days after inoculation that the primary cell membranes are involved in gummous degeneration and that the accumulation of gum between cells forces them apart. Thin-walled cells floating free in the gum mass can be seen at this stage. (Fig. 1, B and C.) The degeneration of the inner lamellae of the walls of these free-floating cells proceeds centripetally until the cell contents are freed and become mixed with the gum matrix. The dissolution and collapse of cells results in the formation of a depression, which marks the site of the lesion. The cuticle, however, remains intact. This is shown by the microscopic appearance of both free-hand and paraffin sections. (Fig. 1, D.) It is shown indirectly by the absence of secondary invaders from isolations from lesions 6 to 7 days old, whose surfaces have been disinfected, since such isolations either have yielded the melanose fungus alone or have remained sterile.

By the time the lesions are 7 days old the differentiation of a phellogen layer has begun in an area several cell layers in advance of gummosis. This is manifest by the formation of cell walls in a plane parallel to the leaf surface. (Fig. 1, F.) The epidermal cells and any of the subepidermal tissues may be involved in the formation of the suberized layer. As a result, a saucer-shaped suberized tissue which completely separates the invaded normal tissues, is formed between them. The growth of this corky tissue proceeds until the tiers of cork are 7 to 12 cell layers in thickness. Meanwhile the normal growth of the healthy tissues beneath the lesions results in everting the corky layer and thus in raising the lesion so that it protrudes about the surface. (Fig. 1, G.) At this stage when the lesions are abundant the affected parts are rough to the touch like sandpaper.

Coincident with the development of the corky layer the tensions on the cuticle result in its rupture and the gum mass on exposure to the air becomes brown and dry. This permits various fungi to penetrate through the fissures. The necrotic tissues of old lesions yield in culture not only the melanose fungus but also species of Colletotrichum, Gloeosporium, Fusarium, Pestalozzia, Cladosporium, and Alternaria.

DISCUSSION

The foregoing observations on penetration and on the presence of the melanose pathogene within the tissues are at variance with previously published accounts. Floyd and Stevens (4) stated that there was apparently no vegetative growth of the fungus within infected tissue in causing the formation of melanose lesions, as evidenced by the fact that stained sections failed to reveal mycelium either within diseased tissues or within adjoining cells. They suggested that the lesions may possibly be caused by some chemical substance or toxic principle that is eliminated by the germination or death of the conidia. In support of this view Stevens (7) reported that dilute lemon juice, when sprayed on young foliage, caused the formation of markings quite typical of melanose. Whatever may be the action of chemicals, the present observations show that the lesions are initiated by the direct penetration of the tissues by the melanose fungus, which accords with infection phenomena in general. However, the observations on the relative abundance of mycelia within melanose lesions, when comparison is made with lesions produced by other pathogenes on other hosts, lead to the conclusion that the hyphae of *Diaporthe citri* are very scarce even in young lesions. The occasional isolation of this fungus from lesions other than melanose markings can most reasonably be interpreted as showing that it may occupy such tissues as a secondary invader.

Microscopic examination discloses the fact that in the formation of melanose lesions the zone of gummous degeneration extends in advance of the mycelium, which shows that this gum is undoubtedly the result of enzymotic action. This observation accords with the well-established fact that the freshly exuded gum in woody plants contains a pectin-dissolving enzyme (5) and that the production of gum is due to enzymotic action. While gum formation in citrus [6] may occur as a response to injury from any cause, as evidenced by its occurrence in connection with such diseases as exanthema, psorosis, and foot rot, the proximate cause in the case of melanose is the pathogene itself through its ability to secrete pectic enzymes.

It is not necessary to assume that the dissolution of the cell walls is due entirely to enzymes secreted by the fungus, since Hodgson (6) has shown in his studies on abscission of leaves and fruits that the pectic enzymes or their appropriate zymogens exist normally within the tissues of citrus.

The manner of the formation of the corky layer presents no novel features, but appears to correspond in all essentials with cork formation of other plants. This layer is therefore to be regarded as wound cork, which is well known to arise as the normal response to traumata.

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[6] The monograph by Butler (*1*), to which the reader is referred for a comprehensive account of gummosis, contains a review of the numerous investigations on this problem. The more recent studies by Fawcett (*3*) further contribute to an understanding of this phenomenon.

SUMMARY

Investigators have hitherto been unsuccessful in isolating *Diaporthe citri* from citrus melanose. During the present study 115 isolations have been secured from a total of 506 trials. Isolations have been made from leaves, twigs, and fruits from lesions that varied in age from 6 days to approximately 9 months. The isolation of *D. citri* from melanose lesions has made it possible to complete Koch's rules of proof of pathogenicity.

Surface disinfection was accomplished by immersion in alcohol and removal of the alcohol by flaming. The lesions were then planted, and as soon as the pathogene had grown from them it was separated from the secondary invaders by means of subcultures.

Direct penetration of conidial germ tubes has been observed. The mycelium is intercellular, and the tissues are disintegrated in advance of the hyphae.

Two phenomena occur in the formation of melanose lesions—gummosis and suberization. Gummosis is the result of enzymotic action, primarily of pectic enzymes, and the melanose fungus itself is able to secrete these enzymes. Suberization is a wound response of common occurrence in citrus and in many other plants.

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